

DNA-interacting Proteins in the Spermiogenesis of the Mollusc *Murex brandaris**

(Received for publication, August 3, 1998, and in revised form, October 13, 1998)

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Sperm chromatin of *Murex brandaris* (a neogastropod mollusc) undergoes a series of structural transitions during spermiogenesis. The DNA-interacting proteins responsible for these changes as well as the mature protamines present in the ripe sperm nucleus have been characterized. The results reveal that spermiogenic nuclear proteins are protamine precursors that are subjected to a substantial number of small N-terminal deletions that gradually modify their overall charge. The composition of mature protamines is remarkably simple in turn, promoting an efficient and extremely tight packaging of DNA. The pattern of spermiogenic chromatin condensation in *M. brandaris* clearly departs from that corresponding to vertebrate chromatin.

The current model for the nuclear changes occurring in spermiogenesis has been essentially drawn from studies on bony fishes and other vertebrates (1–5). The model considers that in early spermatids, histones become acetylated preceding their replacement by a highly basic protein (protamine). During histone displacement, the protamine is found polyphosphorylated, although it undergoes progressive dephosphorylation in the subsequent spermiogenic development. The processes of protamine phosphorylation and dephosphorylation imply a mechanism regulating the interaction with DNA. This mechanism allows for the orderly substitution of protamine for the nucleohistones and for the ensuing binding to DNA. The protamine is a small molecule displaying a very high positive charge density (6, 7). Consequently, the nuclear replacement of histones by protamine induces profound transitions in chromatin structure directly leading to the compactness of the sperm nucleus.

Although these events are well established for some bony fishes and related vertebrates, there is not a universal pattern, or even a “more frequent” model, to account for spermiogenic

processes. Evolution has generated an enormous diversity of both DNA-condensing proteins (8–10) and structural conformations for spermiogenic and/or sperm chromatin (11, 12).

Traditionally, electron microscopy studies have focused on cenogastropod molluscs due to the complexity of their chromatin condensation patterns (13–16). In this regard, *Murex brandaris* is a paradigmatic species (17). Chromatin in the early haploid spermatid displays a somatic-like appearance, which immediately undergoes a peripheral migration, giving rise gradually to granular and fibrillar structures and finally to 18-nm lamellae (18). The nascent lamellar structures are first seen in a disordered arrangement but later become progressively ordered in a regular concentric pattern surrounding the nuclear axis.

The progression of structural changes in chromatin should be paralleled by simultaneous modifications of DNA-protein interactions, concurrently with thermodynamic restrictions on the size of the DNA-protein complexes (19, 20). It was found in a previous study (18) that the histone complement of the immature gonads of *M. brandaris* becomes replaced by a large set of intermediate proteins that lead to the final appearance of three small and simple protamine molecules in the sperm nucleus. It was also observed that these intermediate proteins reacted positively to antibodies elicited against a sperm protamine. In this paper, we report the primary structure of the *M. brandaris* mature protamines and show that one of them (protamine P1) is synthesized as a precursor molecule that undergoes a complex series of partial deletions in its N terminus in conjunction with a single step of dephosphorylation. The final forms of these mature protamines generate an almost extreme packing of sperm DNA.

MATERIALS AND METHODS

Living Organisms—Male specimens of the mollusc *M. brandaris* were collected periodically on the Mediterranean coast of Spain and moved live to the laboratory in cold sea water.

Nuclear Preparation—Either free-flowing sperm cells or gonadal tissue was homogenized separately in ice-cold buffer (0.25 M sucrose, 10 mM MgCl₂, 3 mM CaCl₂, 10 mM Tris-HCl, pH 7.0, 0.1% Triton X-100, 50 mM benzamidin chloride as protease inhibitor) and centrifuged at 3000 × g for 5 min. Crude nuclear pellets were then rehomogenized and resedimented three times in the same buffer. The purified nuclear pellets were next homogenized in 10 mM Tris-HCl, pH 7.0, 20 mM Na₂EDTA, followed by centrifugation and then one more time in the same buffer, omitting the chelating agent. The nuclear sediments thus obtained were used to extract proteins as well as for x-ray diffraction analyses.

Protein Extractions—Proteins were extracted from purified sperm or gonadal nuclei with 0.4 N HCl, precipitated with 6 volumes of cold

* This work was supported in part by Spanish Dirección General Interministerial de Ciencia y Tecnología Grants PB93-1067 (to J. A. S. and M. C.) and PB97-1136 (to L. C.) and by European Economic Community Grant SC1-CT91-0693 (to S. M. and L. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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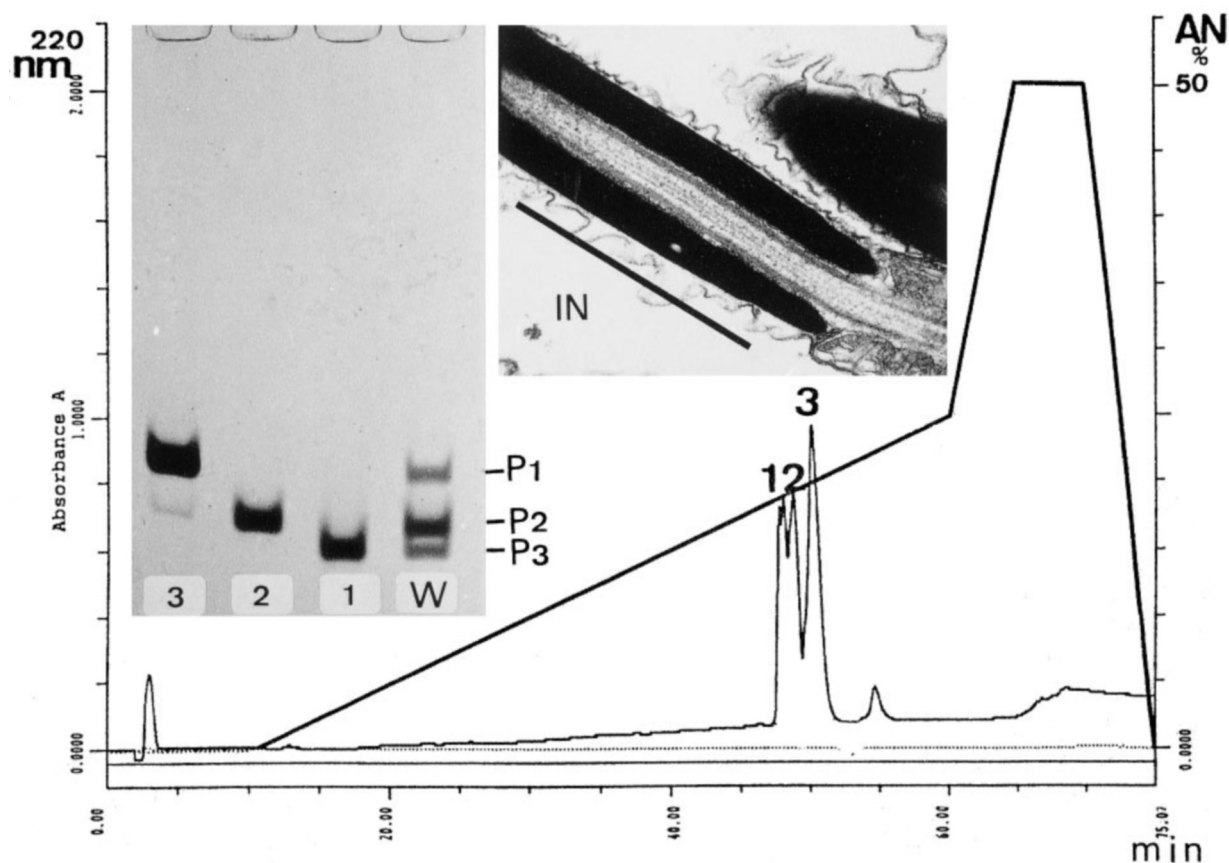


FIG. 1. **Purification of sperm nucleoproteins from *M. brandaris*.** Densitometer tracings of the HPLC chromatographic fractionation of the sperm protamines. Proteins under chromatographic peaks 1–3 were subjected to acid-urea-polyacrylamide slab gel electrophoresis (lanes 1–3, respectively). Lane W corresponds to the total protein complement extracted from purified, ripe sperm nuclei (Protamines 1, 2, and 3). *Inset*, electron microscopy of a ripe sperm head showing the extreme degree of DNA packing (bar, 1 μ m; min, minutes; AN, acetonitrile). Direction of electrophoresis is from top to bottom in this and subsequent figures.

TABLE I
Amino acid composition of *M. brandaris* protamines P1, P2, and P3

Shown are the mol % determined from the compositional analysis (a), corresponding residue contents (b), and number of residues derived from the amino acid sequence (c).

| | P1 | | | P2 | | | P3 | | |
|----------------|-------|-------|----|-------|-------|----|-------|-------|----|
| | a | b | c | a | b | c | a | b | c |
| | mol % | mol % | | mol % | mol % | | mol % | mol % | |
| Lys | 34.4 | 24.8 | 25 | 37.1 | 21.5 | 21 | 38.2 | 20.6 | 21 |
| Arg | 34.0 | 24.5 | 24 | 36.5 | 21.2 | 21 | 36.3 | 19.6 | 19 |
| Ser | 2.0 | 1.4 | 1 | 3.2 | 1.9 | 2 | | | |
| Gly | 26.5 | 19.1 | 19 | 23.1 | 13.4 | 14 | 25.3 | 13.6 | 14 |
| Ala | 1.1 | 0.8 | 1 | | | | | | |
| Cys | 1.9 | 1.4 | 2 | | | | | | |
| Total residues | | | 72 | | | 58 | | | 54 |

acetone, and finally rinsed with acidified acetone (21). On one occasion, nuclear sediments were reduced with 50 mM Tris-HCl, pH 8.8, 2 mM Na₂EDTA, 10 mM dithiothreitol for 1 h at 37 °C under N₂ atmosphere and then alkylated with 12.5 mM iodoacetamide in the same buffer prior to HCl extraction (22, 23).

Preparation of Antibodies and Immunodetection of the Protamine P1 Precursor—Polyclonal antibodies against protamine P1 were prepared as described previously (18) with some modifications. Briefly, outbred New Zealand White female rabbits were multi-injected intradermally with 750 μ g of protamine/animal. The antigen was previously emulsified with Freund's complete adjuvant. The anti-protamine P1 antisera was tested by an enzyme-linked immunosorbent assay with purified protamines.

Western blotting of nuclear proteins from both sperm and gonads was performed according to Harlow and Lane (24). Following acetic acid-urea polyacrylamide gel electrophoresis, proteins were electrotransferred onto nitrocellulose membranes at 500 mA for 1 h at 4 °C in a solution of 0.7% acetic acid, 10% methanol. The membranes were

washed in PBS, 0.1% Tween 20 (PBS/T), blocked with PBS/T containing 0.1% gelatin for 2 h, and incubated with anti-protamine P1 antisera for 2 h at 37 °C. Detection was performed with diluted peroxidase-conjugated anti-rabbit IgG (1:1000) and 4-chloro-1-naphthol as substrate.

Chromatography—Chromatographic resolution of proteins in ion exchange columns was performed on CM-52 cellulose (Whatman). Protein samples were dissolved in 0.2 N NaCl, 50 mM acetate, pH 6.0, and loaded onto the column. Elution was carried out stepwise with 0.2 and 0.6 N NaCl, finally applying a linear gradient of NaCl concentrations from 0.6 to 2.0 N in the same acetate buffer (25). The collected fractions were dialyzed against 5 mM HCl and lyophilized.

For reverse phase HPLC,¹ a C4, 300-Å Delta-Pack column (25 \times 0.46 cm) was used. Proteins were eluted applying a linear gradient of acetonitrile (0–25%) in 0.05% trifluoroacetic acid (26).

¹ The abbreviation used is: HPLC, high pressure liquid chromatography.

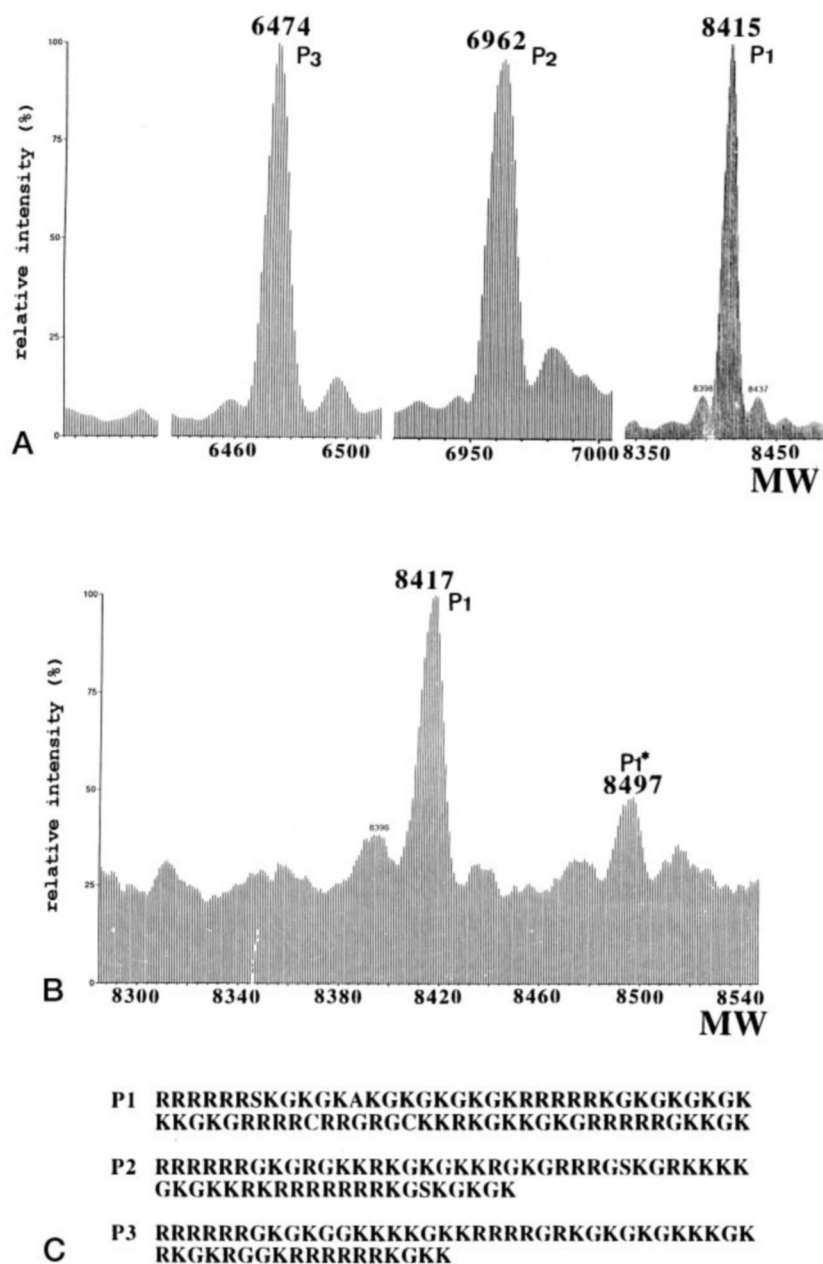


FIG. 2. Primary structure of *M. brandaris* protamines. A, ion spray mass spectrometry of protamines P3 (6474 Da), P2 (6962 Da), and P1 (8415 Da). B, ion spray mass spectrometry of protamine P1 isolated from ripe gonads (gonadal spermatozoa). Note that a fraction of P1 yields a mass of 8497 Da. C, amino acid sequences of protamines P1, P2, and P3 obtained from automated Edman degradation (MW, relative molecular weight).

Electrophoresis—One-dimensional polyacrylamide slab gel electrophoresis was performed according to Panyim and Chalkley (27) with the modifications described by Hurley (28).

Protein Analyses—Amino acid analysis of proteins was carried out after hydrolysis of the samples in 6 N HCl (29). Sequencing of the *M. brandaris* proteins was performed on a Procise Sequenator/ABI-492 (Perkin-Elmer) using the Pulsed Liquid 2HL program. The molecular mass of proteins was determined by ion spray mass spectrometry. Samples were dissolved in 200 μ l of an aqueous solution of 20% acetonitrile, 0.1% HCOOH. Ion spray mass spectra were recorded on a simple quadrupole mass spectrometer API I (Perkin-Elmer), equipped with an ion spray (nebulized-assisted electrospray) source (Sciex, Toronto, Canada). The solutions were continuously infused with a medical infusion pump (model 11, Harvard Apparatus, South Natick, MA) at a flow rate of 5 μ l/min. Polypropyleneglycol was used to calibrate the quadrupole. Ion spray mass spectra were acquired at unit resolution by scanning from m/z 400 to 1200 with a step size of 0.1 Da and a dwell time of 2 min. Ten spectra were summed. The potential of the spray needle was held at +4.5 kV. Spectra were recorded at an orifice voltage of +90 V. A Mac Bio Spec computer program was used to measure the molecular masses of the protein samples. Protein alignments were done using the method of Lipman and Pearson (30).

X-ray Diffraction Analysis—The x-ray diffraction patterns were obtained from samples of nuclei or fibrous complexes (reconstituted complexes). In both instances, samples were sealed in capillaries containing a drop of a saturated salt solution used as a control of the relative humidity. The patterns were recorded with nickel-filtered copper radiation on Kodak film. Either a modified Philips microcamera or a Statton camera (W. R. Warhus, Wilmington, DE) was used.

Complexes of *M. brandaris* protamines with DNA were prepared by mixing both components in a proportion to achieve complete charge neutralization. The mixing buffer used was 2 M guanidine hydrochloride, 1 mM Tris-HCl, pH 8.0, 1 mM EDTA. Mixtures were sequentially dialyzed against solutions of 2, 1, 0.8, 0.4, and 0.2 M guanidine hydrochloride, 1 mM Tris-HCl, pH 8.0, 1 mM EDTA, followed by extensive dialysis against 1 mM Tris-HCl, 1 mM EDTA. Complexes started to precipitate when the guanidine hydrochloride concentration was about 0.8 M. The fibrous precipitates were then pulled with tweezers and allowed to dry under tension in order to yield fibers suitable for x-ray diffraction analysis (31, 32).

Electron Microscopy—Electron microscopy analysis was performed as described previously (18). Either gonadal tissue or sperm cell sediments were fixed in 2.5% glutaraldehyde, 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffer. The samples were

next dehydrated and soaked in Spurr's resin. Sample sections were stained with uranyl acetate and lead citrate and examined under a Hitachi H-600 transmission electron microscope.

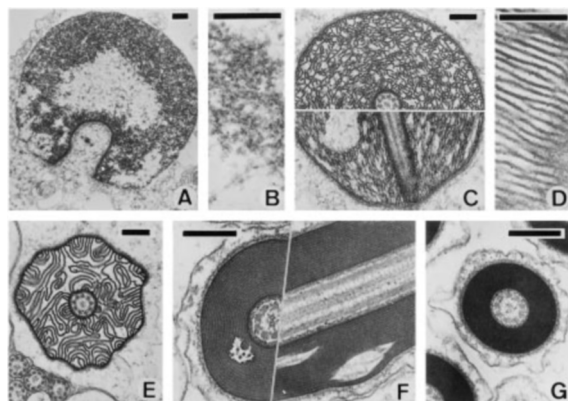


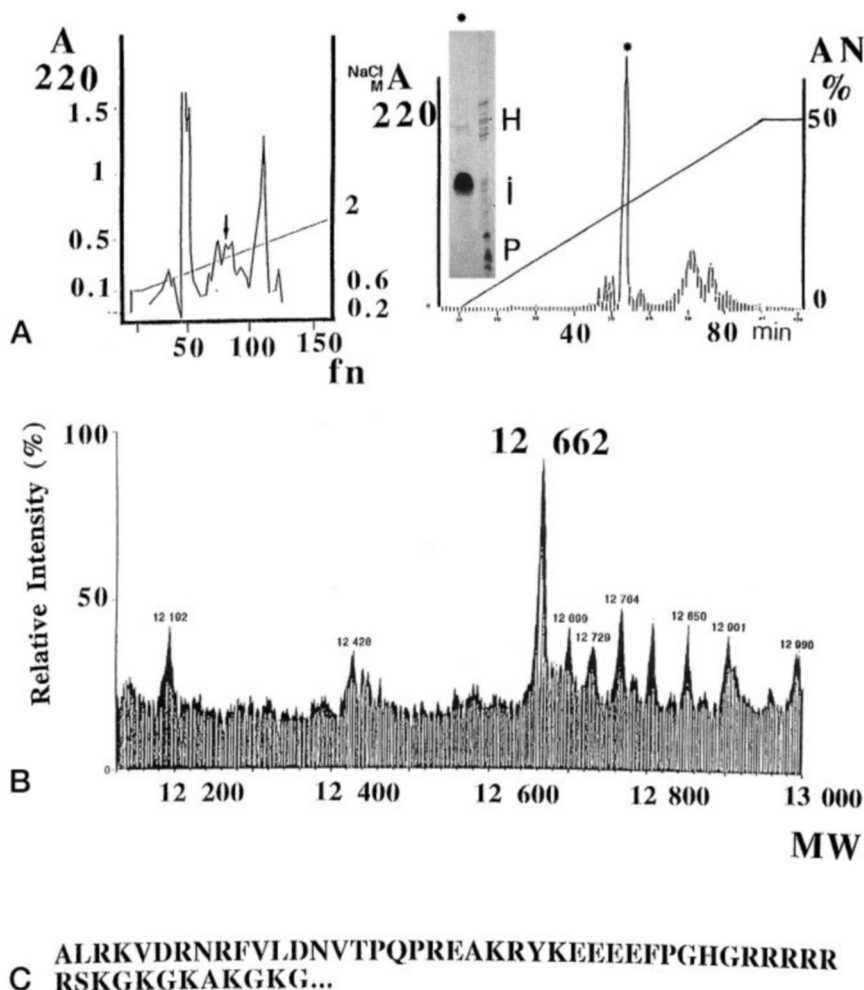
FIG. 3. Transitions in the spermiogenic chromatin of *M. brandaris*. Electron micrographs showing successive stages of nuclear condensation during spermiogenesis in *M. brandaris*, illustrative of the main changes undergone by chromatin. *A* and *B*, fiber-granular structures in early spermatids (*B*, detail). *C*, fibrillate structures in elongating spermatids (transverse (top) and semilongitudinal (bottom) sections; *D*, detail). *E*, transverse view of relatively disordered lamellae in elongated spermatids. *F*, concentric arrangement of chromatin lamellae (transverse (left) and longitudinal (right) sections). *G*, homogeneously condensed chromatin following lamellae coalescence in the ripe sperm nucleus seen in a transverse view. Bars, 250 nm.

RESULTS

Purification of *M. brandaris* Protamines—Sperm cells of *M. brandaris* contain electrodense cylindrical nuclei grooved by axonemes (Fig. 1, inset). Acid extraction of purified nuclei with 0.4 N HCl yielded three basic proteins (Fig. 1, lane *w*). Previous chemical reduction of chromatin prior to extraction did not solubilize any additional proteins. Nucleoprotein components were purified by reverse-phase HPLC eluting in the order P3 → P2 → P1 (Fig. 1). Protamine P1 (peak 3 and lane 3 in Fig. 1) was repurified by reverse-phase HPLC in the same conditions to eliminate a small amount of contaminating P2 protamine.

Analysis of Protamines—The amino acid analysis of *M. brandaris* protamines revealed a set of simple molecules of very basic nature (Table I). P1 is made up of six different aminoacyl types, P2 by only four types, whereas P3 appears to be the simplest protamine known to date, involving just three amino acid residues (Arg, Lys, and Gly). Arginine, lysine, and glycine are the major components of all three protamines (94.9% in P1, 96.7% in P2, and 100% in P3). Another relevant feature revealed by the amino acid analyses was the low proportion of phosphorylatable residues in all of the molecules (2% in P1, 3.2% in P2, and 0% in P3). This is a striking fact if it is considered that phosphorylation and dephosphorylation processes regulate the interactions with DNA in vertebrate protamines. All protamines were repeatedly analyzed by automated Edman degradation with consistent results. Due to the yields and purity of the protein samples together with the powerful methodology employed, it was possible to obtain complete and unambiguous sequences in single sequencing runs (Fig. 2C).

FIG. 4. Purification and characterization of the protein Pr-P1. *A*, CM-cellulose ion exchange chromatography of proteins extracted from nuclei of ripe *M. brandaris* gonads (left). The material of the fraction marked with an arrow was subjected to additional HPLC purification (right). The inset shows the electrophoretic patterns of the gonadal nucleoprotein complement (right) and the purified Pr-P1 protein from HPLC (*). Migration ranges are indicated for core histones (*H*), intermediate proteins (*I*), and protamines (*P*). *B*, ion spray mass spectrometry of the Pr-P1 protein (12,662 Da). *C*, sequence of the amino-terminal region of purified protein Pr-P1 obtained by Edman degradation (initial 52 amino acid residues of the N terminus). The final 17 residues of Pr-P1 sequenced coincide entirely with the leading amino acids of the amino-terminal sequence of protamine P1 (see Fig. 2). AN, acetonitrile.



Molecular masses of protamines P1, P2, and P3 as established by ion spray mass spectrometry were 8416 ± 1 Da, 6962 Da, and 6474 Da, respectively (Fig. 2A). The molecular mass of protamine P1 was also determined in mature gonads containing partially ripe spermatozoa (Fig. 2B). In this case, a molecular mass of 8497 Da was obtained for a fraction of the protamine, consistent with the phosphorylated form ($8417 + 80$ Da). This result suggests that protamine P1 is monophosphorylated in the stages preceding full ripening of the sperm and is coincident with the presence of only one serine residue in the P1 molecule.

The analytical results so far reported are wholly congruent. First, the molecular masses derived from the amino acid analyses (P1, 8415 Da; P2, 6960 Da; P3, 6473 Da) are totally coincident with the masses determined. Second, the number of amino acid residues obtained from the compositional analyses is practically identical to that afforded by the primary structures (see Table I).

Protamine P1 Precursors in *M. brandaris*—It is relevant to note the simplicity of the *M. brandaris* protamine sequences whose organization will be dealt with later. Protamine P3 appears exclusively constituted of Arg, Lys, and Gly amino acids, while both P1 and P2 contain a few additional residues. This structural simplicity together with the low proportion of

phosphorylatable residues (only three serines out of 184 amino acid residues) contrasts with the elaborated forms of spermiogenic chromatin condensation illustrated in Fig. 3. We have previously shown that unripe spermiogenic nuclei of *M. brandaris* contain a substantial number of proteins with an electrophoretic mobility intermediate between histones and protamines in denaturing polyacrylamide gels (18). This abundant subset of intermediate proteins disappears in very advanced stages of spermiogenesis and becomes wholly absent from ripe sperm nuclei. Considering both the electrophoretic behavior and amino acid composition of these intermediate proteins, we surmised that they might direct the complex transitions in the condensation of the *M. brandaris* spermiogenic chromatin, modulating the interaction with DNA through a series of post-translational intranuclear modifications and acting as precursors of the ripe sperm protamines.

In order to verify the preceding assertion these proteins were analyzed in detail. First, the largest intermediate molecule Pr-P1, displaying the slowest electrophoretic mobility, was purified, and the sequence of its N terminus comprising the initial 52 amino acid residues was determined by automated Edman degradation together with the assessment of its molecular mass by ion spray mass spectrometry (Fig. 4). Moreover, purified Pr-P1 protein reacted positively with anti-protamine P1 antisera (Fig. 5, lane e). Table II shows the actual amino acid composition of the putative precursor molecule and its comparison with the composition estimated under the assumption that the molecule is a true precursor of protamine P1. The results indicate that this intermediate protein (Pr-P1) indeed corresponds to a monophosphorylated precursor of protamine P1. Thus, the molecular mass of the monophosphorylated form of protamine P1 (8497 Da in Fig. 2) plus that corresponding to the 35 initial residues of the N terminus of Pr-P1 yielded a value (12,657 Da) almost identical to the molecular mass of Pr-P1 obtained by ion spray mass spectrometry (12,662 Da). In addition, the determined amino acid composition is very similar to the composition estimated (Table II). Finally, the last 17 sequenced residues of Pr-P1 are identical to the 17 initial amino acid residues of P1. The presence of an alanine in position 48 of Pr-P1 confirms that this molecule truly encompasses protamine P1, since both P2 and P3 lack the former residue.

It can be seen in Fig. 5 that the anti-protamine P1 antiserum reacts positively with the bulk of intermediate proteins present in spermiogenic nuclei (see lane b). This result suggests that these proteins do contain also the protamine P1 sequence and therefore can be considered precursor molecules. To confirm this assumption, the material under the individual peaks in Fig. 4A was subfractionated by HPLC, and the resulting proteins were directly sequenced by automated Edman degradation.

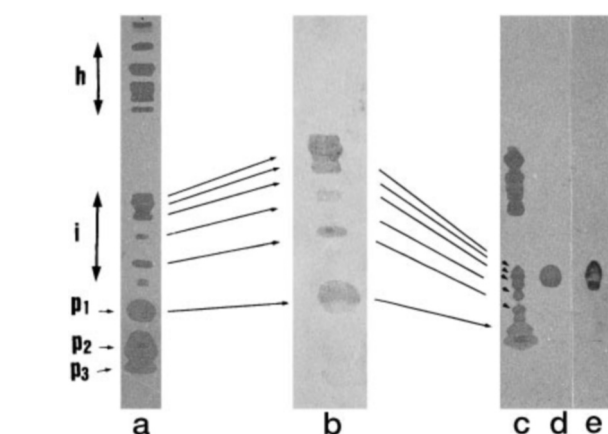


FIG. 5. Immunoreactivity of protein Pr-P1 to anti-P1 antisera. Western blot analysis of *M. brandaris* proteins with polyclonal antibodies after acetic acid-urea-polyacrylamide gel electrophoresis is shown. Lane a, blot of the nucleoproteins extracted from whole gonads stained with Amido Black following electrotransfer onto nitrocellulose filter. Lane b, same strip as in A immunoreacted with anti-P1. Lanes c and d, electrophoretically resolved gonadal nucleoproteins (lane c) and purified protein Pr-P1 (lane d), blotted to membranes and stained with Amido Black. Lane e, strip containing purified protein Pr-P1 reacted with anti-P1 antibodies. Migration ranges are indicated for histones (h), intermediate proteins (i), and protamines (p).

TABLE II
Compositional analysis of Pr-P1

Shown are the amino acid composition (mol %) determined for protein Pr-P1 (a) and estimated composition considering Pr-P1 a direct precursor form of protamine P1 (b).

| a | | | b | | |
|-------|------|--|-------|-----------------|------|
| mol % | | | mol % | | |
| Lys | 26.1 | | 26.2 | | |
| His | 1.4 | | 0.9 | | |
| Arg | 27.1 | | 27.1 | | |
| Asx | 4.0 | | 3.8 | | |
| Asp | | | 1.9 | | |
| Asn | | | 1.9 | | |
| Thr | 1.2 | | 0.9 | | |
| Ser | 1.3 | | 0.9 | | |
| Glx | 6.6 | | 9.3 | | |
| Glu | | | 8.4 | | |
| Gln | | | 0.9 | | |
| | | | Pro | 2.9 | 2.8 |
| | | | Gly | 20.8 | 19.6 |
| | | | Ala | 2.3 | 2.8 |
| | | | Cys | 0.9 | 1.9 |
| | | | Val | 2.1 | 2.8 |
| | | | Met | | |
| | | | Ile | | |
| | | | Leu | 1.2 | 0.9 |
| | | | Tyr | 0.2 | 0.9 |
| | | | Phe | 1.9 | 1.9 |
| | | | Trp | ND ^a | |

^a Not determined.

FIG. 6. Primary structure of precursor forms of *M. brandaris* protamine P1. Amino-terminal sequences obtained from intermediate nucleoproteins during the spermiogenesis of *M. brandaris*, Pr-P1 and Pr8 to Pr1.

| | |
|--------------|---|
| PR-p1 | ALRKVDRNRFVLDNVTPQPREAKRYKEEEFPGHGRRRRRRSKGKGAKGKGK |
| PR(8) | DRNRFVLDNVTPQPREAKRYKEEEFPGHGRRRRRRSKGKGAKGKGKGK |
| PR(7) | NRFVLDNVTPQPREAKRYKEEEFPGHGRRRRRRSKGKGAKGKGKGKGRRR |
| PR(6) | FVLDNVTPQPREAKRYKEEEFPGHGRRRRRRSKGKGAKGKGKGKGRRRR |
| PR(5) | NVTPQPREAKRYKEEEFPGHGRRRRRRSKGKGAKGKGK |
| PR(4) | KRYKEEE |
| PR(3) | YKEEEFPGHGRRRRRRSKGKGAKGKG |
| PR(2) | KEEEFPGHGRRRRRRSKGKGAKGKGKGKGRRRRRKGKGK |
| PR(1) | EEFPGHGRRRRRRSKGKGAKGKGK |
| P1 protamine | RRRRRSKGKGAKGKGKGKGRRRRRKGKGKGK./ /GK |

TABLE III
Precursor forms of protamine P1

Shown are the number of amino-terminal residues sequenced for each intermediate molecule (NS), molecular masses determined by ion spray mass spectrometry (Mwo), and molecular mass estimated under the assumption that these molecules are precursors of the monophosphorylated form of protamine P1 (Mwe) (see also Fig. 6).

| Protein | NS | Mwo | Mwe |
|---------|----|-----------------|--------|
| | | Da | Da |
| Pr-P1 | 52 | 12,662 | 12,657 |
| Pr8 | 50 | 12,092 | 12,090 |
| Pr7 | 54 | 11,820 | 11,819 |
| Pr6 | 52 | 11,550 | 11,549 |
| Pr5 | 40 | 11,074 | 11,075 |
| Pr4 | 7 | ND ^a | 10,082 |
| Pr3 | 29 | ND | 9798 |
| Pr2 | 43 | ND | 9635 |
| Pr1 | 25 | 9248 | 9249 |

^a ND, not determined.

tion. In some cases, protein yields allowed for additional determinations of the molecular mass by ion spray mass spectrometry (Table III). The sequences obtained (Fig. 6) clearly show that the intermediate proteins analyzed arise from the gradual trimming of the N-terminal residues of protein Pr-P1. Moreover, the molecular weights determined (Table III) indicate that these molecules are monophosphorylated. Nine major precursor forms have been identified; however, the presence of additional minor components cannot be discounted. These results show that the *M. brandaris* Pr-P1 precursor is processed by means of serial deletions each involving one or a few more amino acid residues. Although this processing mechanism is not common to other molluscs (33), it stands comparison with the processing of the P2 precursor protamine in some mammals (34, 35).

X-ray Diffraction Analysis of *M. brandaris* DNA-Protamine Complexes—To determine the capacity of *M. brandaris* protamines to compact DNA, protamine-DNA complexes were subjected to x-ray diffraction analysis. Two sets of experiments were carried out. In the first series, x-ray diffraction patterns were obtained from purified sperm nuclei, whereas in the second the analyses were performed with reconstituted complexes of DNA with purified *M. brandaris* protamines (see “Materials and Methods”). Nuclei or reconstituted complexes were analyzed at different values of relative humidity. Fig. 7 shows the x-ray diffraction patterns obtained from reconstituted complexes at 76% relative humidity (Fig. 7A) and from sperm nuclei at 92% relative humidity (Fig. 7B). The complex in Fig. 7A may be interpreted as a B form of DNA packed in an orthorhombic lattice with two molecules per unit cell, with approximate cell parameters as follows: $a = 33.2 \text{ \AA}$, $b = 24.1 \text{ \AA}$, and $c = 33.4 \text{ \AA}$. The values reported in Table IV show that distances between DNA strands observed in reconstituted complexes and sperm nuclei are highly coincident at each value of

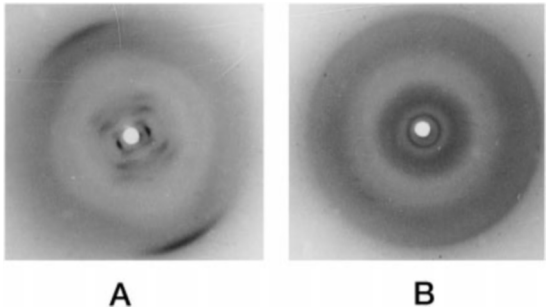


FIG. 7. DNA packing by *M. brandaris* protamines. X-ray diffraction patterns of reconstituted complexes of DNA with *M. brandaris* protamines P1, P2, and P3 at 76% relative humidity (A) and whole nuclei at 92% relative humidity (B).

relative humidity.

These experiments show that DNA becomes packaged by *M. brandaris* protamines in a very tight and compact manner as deduced from the distance between axes of the neighbor DNA strands, which varies between 19.5 and 22.8 Å (see Table IV). These values are similar to those found in squid nucleoprotamine, which contains 79% arginine (36). It is likely that the charge distribution and large percentage of glycine (an amino acid with a minimal volume) in the protamines of *M. brandaris* generate a compactness of the DNA to such extremes that the resulting volume of the mature sperm nucleus becomes comparable with that of the DNA alone when considered as a cylinder of 20 Å in diameter.

DISCUSSION

Three protamines of a very simple composition (P1, P2, and P3) are present in the ripe sperm nuclei of the mollusc *M. brandaris*. One of them (P3) is the simplest protamine known to date, being made up of only three types of amino acids (Gly, Lys, and Arg) and completely lacking phosphorylatable residues. The most relevant features of these three molecules are their extreme basicity and the presence of arginine clusters interspersed with very rich GK tracts. Arginine clusters have been held responsible for the cooperative interaction of protamines with DNA (37), but GK-rich regions are absent in other known protamines. Among the *M. brandaris* protamines, the GK-alternating residues are particularly evident near the N terminus of protamine P1 (see residues 8–22 in Fig. 2). It is worth indicating that some other protamines contain substantial clusters of alternating basic/nonbasic residues close to their N termini, notably the (RS)_n repeat (5, 45). Interestingly, a (RS)_n motif adjacent to a tract of basic amino acid residues has been observed in some splicing factors, having been implicated in intranuclear location (38). The repeating GK dipeptide might also represent a novel protamine-DNA element, although further structural studies are required to unambiguously establish its specific role in the highly efficient and orga-

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